REMARKS

By this amendment, claims 1, 16, 29, and 30 have been cancelled; claims 14 and 15 have been amended; and claims 46-70 have been added. Thus, claims 14, 15, and 46-70 are pending. Applicants cancelled claim 1 without prejudice to further prosecution of the remaining epitopes, solely to speed allowance of the present claims.

Support for the new claims is derived from the specification from at least the following locations: Claims 14-15: page 2, lines 18-35; Claims 46, 47 and 57: page 18, lines 27-32; Claims 48-57: page 2, lines 3-7, generally; Claims 50 and 67: page 20, lines 1-3; Claims 51-56 and 61-63: page 2, lines 9-17, and pages 9 and 10; Claims 57 and 64: page 15, lines 13-15; Claim 58: page 17; Claims 65-66: page 20, lines 1-3; and all claims, pages 17-21.

Support for the specification amendments of the sequence of SEQ ID NO: 4, found on page 2, line, 22 and in the chart on page 14, is found in attached Exhibit A. The article of Exhibit A, Selection of Continuous Epitope Sequences and Their Incorporation..." by Qiu, et al. is incorporated by reference on page 1 of the present specification. The article clearly shows on page 321, last sentence of column one, and page 323 in Table 1, that the list of synthesized epitope peptides, particularly SEQ ID NO: 4, was intended to be depicted as is shown in the present amendments. Thus, the sequence amendment is pure typographical in nature and is not a substantive change.

The Office Action objected to a number of informalities in the specification, all of which have been corrected as requested. The Action asks what "guluronic" is on page 6, line

30. Applicants intended to use this term. Guluronic acid is a polymer that makes up alginate. (See Exhibit B)

Applicants are also concurrently submitting a sequence listing pursuant to 37 CFR 1.821(d) and have added SEQ ID NOS: 1-7 in the specification to indicate the seven epitope amino acid sequences. Objections to claims 29 and 30 are now moot because these claims have been cancelled. Applicants have amended claims 14 and 15 so the antecedent basis rejection is also no longer applicable.

The Office Action rejected the claims relating to vaccines as not enabled under 112(1). Again, without prejudice to further prosecution, Applicants have amended the claims to include only what the Action specifically states is enabled, namely, using the polypeptide as a component of a composition that binds to antibodies of Lyme disease patients.

With respect to the double patenting rejection, Applicants have added a variation of the kit claims from copending App. No. 09/982264. The fact that the Action would require a terminal disclaimer for an obviousness-type double patenting rejection is further proof that the new claims are related to the pending claims. Applicants are submitting a preliminary amendment in the 09/982264 case, which should eliminate the requirement for a terminal disclaimer.

Applicants believe this addresses all issues raised in the office action. It is submitted that the claims are allowable, and issuance of a Notice of Allowance is respectfully requested. Applicants do not believe any fees are associated with this paper, but if necessary, the Commissioner is authorized to charge any fees required by the filing of these papers, and to credit any overpayment to Perkins Coie's Deposit Account No. 50-2586. If Applicants can do anything more to expedite this application, Applicants ask the Examiner to contact the undersigned at (310) 788-9900.

Respectfully submitted,

PERKINS COIE LLP

Dated: January 23, 2004

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Exhibit A

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Selection of Continuous Epitope Sequences and Their Incorporation into Poly(Ethylene Glycol)–Peptide Conjugates for Use in Serodiagnostic Immunoassays: Application to Lyme Disease

Abstract: Continuous epitope sequences were selected from immunogenic Bb proteins by epitope mapping. The identified epitope sequences were synthesized by solid phase peptide synthesis and purified by high performance liquid chromatography. Each epitope was conjugated individually to a multifunctional poly(ethylene glycol) (PEG) carrier. The result PEG–peptide conjugates were used as antigens in ELISA for diagnosis of Lyme disease. The results showed that the defined epitope peptides were Lyme disease specific and could be used in a format of PEG-peptide conjugate as the antigen to achieve improved sensitivity and specificity. © 2001 John Wiley & Sons, Inc. Biopolymers (Pept Sci) 55: 319–333, 2000

Keywords: Lyme disease; epitope peptides; poly(ethylene glycol); polymer–peptide conjugate; serodiagnostic immunoassay

INTRODUCTION

Lyme disease (LD) is an infection caused by the spirochete *Borrelia burgdorferi* (*Bb*), which is transmitted to the human body by infected deer ticks.[1–3] LD usually responds very well to antibiotic therapy, especially in its early stages. Early diagnosis followed by an appropriate course of antibiotic treatment can prevent progression to complex manifestations of late stage disease and eventually cure the disease. However, the diagnosis of early LD, without the presence of a distinctive skin lesion, erythema migrans (EM), is

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definitely not a clear-cut decision to make because the clinical symptoms of LD are constitutional and nonspecific. If EM is present, particularly for those patients with tick bite history, the diagnosis of LD can be made confidently. Patients who do not present acutely with the unique EM marker will obviously have less chance for a prompt, definitive diagnosis. Even patients with EM sometimes may not be correctly diagnosed because either EM can resolve by itself without any treatment and/or patients cannot recall any tick bite. Indeed, the disease often develops insidiously and is not diagnosed until its late stage with complex manifestations.

Until now, an accurate and detailed medical history and a careful physical examination still form the basis of arriving at a correct LD diagnosis. Most of the currently available laboratory tests for LD lack sufficient sensitivity and specificity to be relied upon for diagnosing early LD. The current recommendation from the Centers for Disease Control and Prevention (CDC) for LD serological diagnosis is first to test with an immunoassay titration, such as an ELISA, then to test all positive and equivocal results with Western blot analysis for confirmation. This two-test approach can be useful for late stage LD when all the important antigenic proteins have been expressed and the antibodies against them have been fully developed. However, it is not accurate enough for early stage LD diagnosis because both methods rely on the detection of specific serum antibodies, and many early stage patients fail to develop a specific serum antibody level high enough for currently used diagnostic tests.

Both ELISA and Western blot analysis use extracts of sonicated whole *Bb* spirochete as the antigen source. It has been found that the *Bb* spirochete contains at least 30 different proteins, including flagellin protein (41 kD), outer surface protein A (OspA, 31–32 kD), B (OspB, 34–36 kD), C (OspC, 21–23 kD), P39 (39 kD), and P83 (83 kD).[4] When using such a mixture of different proteins to detect serum antibodies, it is quite possible that antibodies against other organisms, most notably spirochetes, can cross-react with *Bb* spirochete proteins, giving false positive results. ELISAs performed with a single purified *Bb* spirochete protein, in the form of either a native or a recombinant protein have demonstrated improved specificity with comparable sensitivity. However, cross-reactions still happen because these proteins contain many epitopes and some of them can still interact with antibodies against other organisms. The proteins of other organisms do not need to be identical to those of *Bb* spirochete to cause cross-reactions, rather, the cross-reaction can occur if individual epitopes within these proteins are identical or similar.

To solve this common problem in diagnostic assays of many infectious diseases, we propose to use defined individual epitopes composed of small peptides without adjacent sequences. Multiple copies of epitope will be conjugated to a polyethylene glycol carrier and used as the antigens for LD serodiagnostic immunoassay.

RESULTS AND DISCUSSION

Epitope Selection

Antigens comprise discrete epitopes that can be recognized by antigen-specific receptors on lymphocyte surface to elicit immune responses. These antigenic epitopes can interact specifically with the antigen-binding sites of the induced antibodies. A particular antigen can have several different epitopes or repeated epitopes. Antibodies are specific for the individual epitope rather than the whole antigen molecule and each antibody can generally only bind to just one antigen, or more accurately one epitope of that antigen. Theoretically, in order to increase specificity of an antibody-detecting assay, it would be preferable to use only defined epitopes without adjacent regions rather than entire antigens, which may share epitopes with other antigens and cause nonspecific interactions with antibodies against those antigens.

Epitopes are essentially polypeptides with lengths ranging from several amino acids to several dozens of amino acids, which can be continuous or noncontinuous in the protein sequence. The selection of epitopes from an antigenic protein can be achieved by epitope mapping the entire sequence of the corresponding protein. Geysen et al. did pioneering work on epitope mapping in the middle of 1980s by synthesizing in parallel a series of peptides on polyethylene pins based on the known protein sequence.[25] Our group has previously identified several LD specific epitopes from *Bb* spirochete proteins using this pin method.[5,6] In this study, we use a simpler epitope screening method, SPOTs, to select epitope sequences from another LD specific and highly conserved *Bb* protein, P39, which is considered to be an important immunogenic protein at the early stages of LD.[7,8]

The SPOTs membrane is made of cellulose and preactivated at individual spots in a format of 8 × 12 spots on the membrane. To activate the cellulose membrane, the hydroxyl groups on the membrane are first transformed into primary amino groups, then the Fmoc-β-alanine active ester (Fmoc: 9-flourenylmethyoxycarbonyl) is added onto the preselected spots to carry out coupling reaction so that only primary

```
1          10         20         30         40         50
MNKILLLILL ESIVFLSCSG KGSLGSEIPK VSLIIDGIFD DKSFNESALN

51         60         70         80         90         100
GVKKVKEEFK IELVLKESSS NSYLSDLEGL KDAGSDLIWL IGYRFSDVAK

101        110        120        130        140        150
VAALQNPDMK YAIIDPIYSN DPIFANLVGN TFRAQEGAFL IGYIAAKLSK

151        160        170        180        190        200
TGKIGFLGGI EGEIVDAFRY GYEAGAKYAN KDIKISTQYI GSFADLEAGR

201        210        220        230        240        250
SVATRMYSDE IDIIHHAAGL GGIGAIEVAK ELGSGHYIIG VDEDQAYLAP

251        260        270        280        290        300
DNVIISTTKD VGRALNIFTS NHLKINTFEG GKLINYGLKE GVVGFVRNPK

301        310        320        330
MISFSLEKEI DNLSSKIINK EIIVPSNKES YEKFLKEFI
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FIGURE 1 Amino acid sequence of 39 kD *Bb* spirochete protein, P39.[10]

amino groups located on these spots are capped with Fmoc-β-alanine. After acetylation of the membrane, all free primary amino groups are permanently blocked and could not react during SPOTs synthesis steps. The primary amino groups are present only at the spot areas after removing the Fmoc protecting groups and are available for further coupling of other amino acids. The SPOTs membrane provided by Genosys has been coupled with a second β-alanine on the spots to provide an 8-atom linker between the membrane and the peptides. In some cases that require more flexible peptides and less steric hindrance, one can couple one or two more β-alanine on the spots to serve as longer linkers. Fmoc-amino acid active esters used in this method allow a direct condensation between the C-terminal COOH and N-terminal NH_2 groups of two amino acids without using any coupling reagents. Alternatively, the coupling reaction can also be carried out by using regular Fmoc-amino acids with a preactivation step by DIPC/HOBt (1,3-Diisopropylcarbodiimide/1-hydroxybenzotriazol hydrate) reagents and the coupling efficiency has been proved to be at least as good as the active ester method.[9]

The 39 kD protein (P39) consists of 339 amino acids and its amino acid sequence has been deduced from its gene nucleotide sequence (Figure 1).[10] Based on its sequence, a total of 83 10-mer peptides with a 6 amino acids overlap and a 4 amino acid offset were synthesized on SPOTs membrane. Two control peptides were included in the membrane synthesis: one is the flagellin peptide (FLA) with a sequence of VQEGVQQEGAQQP; another one is the OspC 10 peptide with a sequence of PVVAESPKKP. Both control peptides are known to be reactive to the antibodies against LD spirochetes.[5,11–13] Since bromophenol blue is extremely sensitive for detecting free amines, it is used to label the initial spots and to monitor the completion of the coupling reactions in each coupling cycle (Figure 2). The N-terminus of each peptide was acetylated so the charge associated with a free terminal amino group was removed, thus to mimic the real environment in the protein sequence. After side-chain deprotection, these peptides were ready for epitope probing by SPOTs analysis.

When performing the peptide screening analysis on a SPOTs membrane for the first time it is important to determine any nonspecific reactions of the peptides with the secondary antibody. Both LD sera and normal control sera were tested with the synthesized peptides on the membrane to identify epitope candidates that specifically react with LD sera but not react with normal control sera. The membrane was first blocked with TBS-blocking buffer (TBS: Tris buffered saline) overnight before analysis to ensure a low background. To test the possible nonspecific reaction of the peptides with the secondary antibody, the membrane was incubated directly with the diluted β-galactosidase conjugated antibody, then continued to the color development steps. Under the specified testing conditions, no nonspecific reaction was observed for all synthesized peptides. For SPOTs analysis, diluted serum antibodies were first incubated with the blocked membrane, then with β-galactosidase conjugated secondary antibody solution. The color was developed by signal development solution containing 5-bromo-4-chloro-3-indoyl-D-galactopyranoside. Figure 3 shows that several spots reacted with LD sera

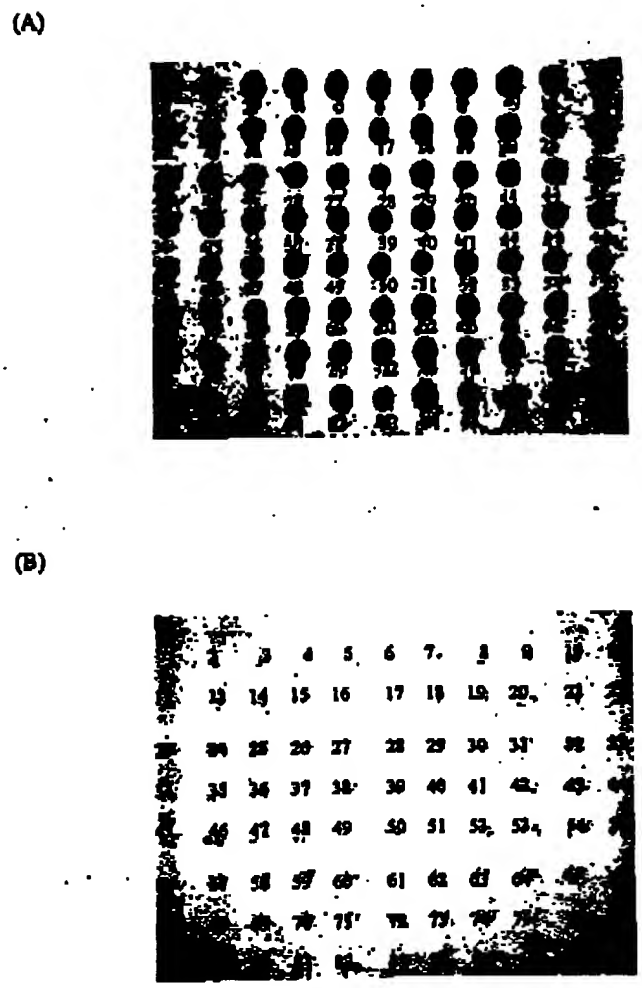


FIGURE 2 Photographs of SPOTS membrane (A) before and (B) after peptide synthesis. In A, all spots gave a blue color before coupling reaction due to the presence of free amines. In B, the blue color disappeared after the coupling reaction due to the absence of free amines.

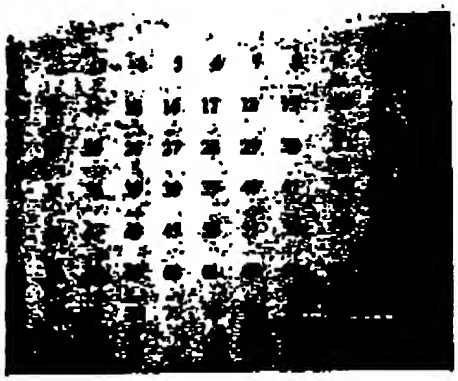
but did not react with normal control sera. Spots 31–34, especially spots 33 and 34, reacted strongly with all three tested LD sera and did not react with the normal serum. The sequence corresponding to those two spots, GMTFRAQEGAFLTG, was selected as the epitope candidate for further study. As expected, the two control peptides reacted with all LD sera but not with the normal control serum.

Synthesis and Characterization of Epitope Peptides

Seven epitope peptides were selected to test their use for LD diagnosis (Table I). Besides the P39 epitope peptide identified by SPOTs method in this study, a central region of the immunodominant Bb flagellin protein, often reactive with LD sera but not reactive with non-LD sera, had been identified previously[11,12] and the optimal peptide sequence was determined using a pin synthesis library.[5] The C-terminal peptide of the outer surface protein C (OspC 10) was chosen as an epitope of this study based on recent publications.[13,14] Two epitope peptides from P83 (P83-1 and P83-3) and another two peptides from the outer surface protein C (OspC-1 and OspC-3) were selected based on an epitope screening study previously conducted in our lab using the PIN method.[5,6]

In order for the conjugation of epitope peptides, we propose to use thiol-specific chemistry under mild conditions. The easiest strategy for peptide conjugation is to add an extra amino acid on either the amino or carboxyl terminus of the peptide to allow one-site

(A)



(B)

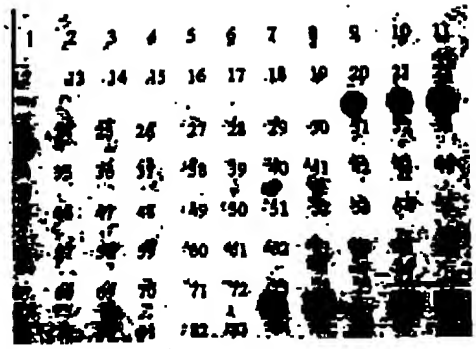


FIGURE 3 Photographs of SPOTS membrane after detecting (A) negative and (B) positive Lyme disease serum sample. In A, no spots reacted with negative serum sample. In B, spots 31–34 reacted with positive serum sample; spots 84, 85, and spots 87, 88 are the control peptides, FLA and OspC-10, respectively.

coupling to the carrier. In our study design, a cysteine residue, followed by two β-alanine residues, was incorporated at the C-terminus of each epitope peptides during solid phase peptide synthesis. Putting two more β-alanine residues between the conjugation anchor, cysteine, and the epitope peptides should generate further flexibility of the linear peptides, and therefore may help them to adopt the optimal confor-

Table I List of Synthesized Epitope Peptides

Peptide	Sequence	MW
FLA, AA 211–223	VQEGVQQEGAQQP-(β-A)(β-A)C	1639.8
OspC2, AA71–86	EIAAKAIGKKIHQNNG-(β-A)(β-A)C	2274.3
OspC3, AA104–118	ISTLIKQKLDGLKNE-(β-A)(β-A)C	2282.3
OspC10, AA198–207	PVVAESPKKPE-(β-A)(β-A)C	1762.7
P83-1, AA296–310	DKKAINLDKAQQKLD-(β-A)(β-A)C	2310.3
P83-3, AA431–442	ITKGKSQKSLGD-(β-A)(β-A)C	1843.8
P39, AA129–142	GMTFRAQEGAFLTG-(β-A)(β-A)C	2067.9

mations for stronger antibody binding. The N-terminus of all epitope peptides was capped with long chain biotin to serve two purposes simultaneously. The first purpose is to remove the charge associated with the free amino group of the N-terminus, thus to mimic the real environment in the natural protein sequence. The second purpose is to use the biotin as the detection label for biotin–avidin binding in ELISA. Long chain biotin was selected to reduce any possible conformational hindrance for high-avidity biotin–avidin binding. The crude peptides were purified by reverse phase high performance liquid chromatography (HPLC) under acidic condition [0.1% triflouroacetic acid (TFA)], because cysteine was incorporated in all epitope peptides for conjugation purpose and the availability of free thiol groups in cysteine is critical for conjugating epitope peptides onto PEG copolymer backbone. The acid condition can help to prevent or minimize the oxidation of the free thiol groups. After HPLC purification, the tubes containing the epitope peptides were flushed with an argon stream, capped, wrapped with parafilm, and stored dry in the refrigerator (+4°C). The purified epitope peptides were characterized by amino acid analysis and mass spectrometry.

Synthesis and Purification of PEG–Aspartic Acid Copolymers

Poly(ethylene glycol) (PEG) itself contains only two functional groups on both ends. In order to obtain a polymer system with multiple functional groups capable of carrying epitope peptides, the amino group protected aspartic acid was selected to copolymerize with diamino-PEG to form a new alternating copolymer. After removing t-Butyloxycarbonyl (Boc) protecting groups, the primary amine became available in the PEG copolymer pendant chain for peptide conjugation. The monomer α,ω-diamino-PEG (NH_2-PEG-NH_2 MW 3400 Da) was chosen to prepare the PEG copolymer because it was commercially available from Shearwater Polymers or Fluka. Other α,ω-diamino-PEG monomers with various molecular weight can be obtained by custom order or synthesized according to published procedure.[15] A carbodiimide-mediated direct polycondensation was successfully employed to copolymerize α,ω-diamino-PEG (NH_2-PEG-NH_2, MW 3400 Da) with Boc-protected aspartic acid (Boc-Asp-OH) at room temperature. The two-component catalyst system, 4-(dimethyl amino)-pyridine (DMAP)/p-toluenesulfonic acid (PTSA), was essential to achieve a high degree of polymerization.[16] The protecting Boc groups on the polymer pendant chains were removed by TFA treatment without degrading the PEG copolymer as judged by size exclusion chromatography (SEC) measurements. The result PEG-aspartic acid copolymer, containing one primary amino group for each repeating unit was used to prepare PEG–peptide conjugates.

Preparation of PEG–Peptide Conjugates

The success of developing a specific and sensitive immunoassay largely depends on the strength of antigen–antibody binding and the stability of the complex formed between the antigen and the antibody. The strength of antigen–antibody binding could be measured by affinity, which is an intrinsic property of an antigen for a given antibody. Selecting an epitope peptide requires the ability to identify a peptide sequence that can bind disease-related antibodies specifically and with high affinity. On the other hand, the stability of complex between antigen and antibody is measured by avidity, which is determined by three factors: the intrinsic affinity of the antibody for the antigen, the valency of the antibody and antigen, and the geometric arrangement of the interacting components.[17]

The sensitivity of an immunoassay relies on having enough of each antigen immobilized on the plate and on having the right orientation and conformation of the adsorbed antigens, the epitope peptides alone without appropriate modification are seldom used directly as antigens in an immunoassay for several reasons. First, since the physical adsorption of antigens to the ELISA plates depends mainly on weak van der Waals forces and hydrophobic interactions, the molecular weight of the antigen is critical for its adherence. The epitope peptides are usually too small to be effectively immobilized onto the plate surface to achieve an amount sufficient for accurate antibody detection.[18] Second, even those actually adsorbed epitope peptides are most often altered or inactivated through direct adsorption to the plates, leading to a substantially underestimated antibody level in sera.[19] Third, since affinity of epitope peptides to antiprotein antibodies can be 10^2- to 10^3-fold weaker than that of the protein counterpart,[20] the interaction between a single epitope peptide and serum antibody might not be strong enough to endure the required vigorous washing steps in an immunoassay. Therefore, epitope peptides must be modified in such a way that they can adsorb firmly onto the surface of the plate (directly or indirectly) and assume the right orientation and conformation to obtain a strong antigen-antibody binding.

One widely used approach is to couple multiple copies of small epitope peptides to large carriers, thereby enhancing indirect immobilization of the

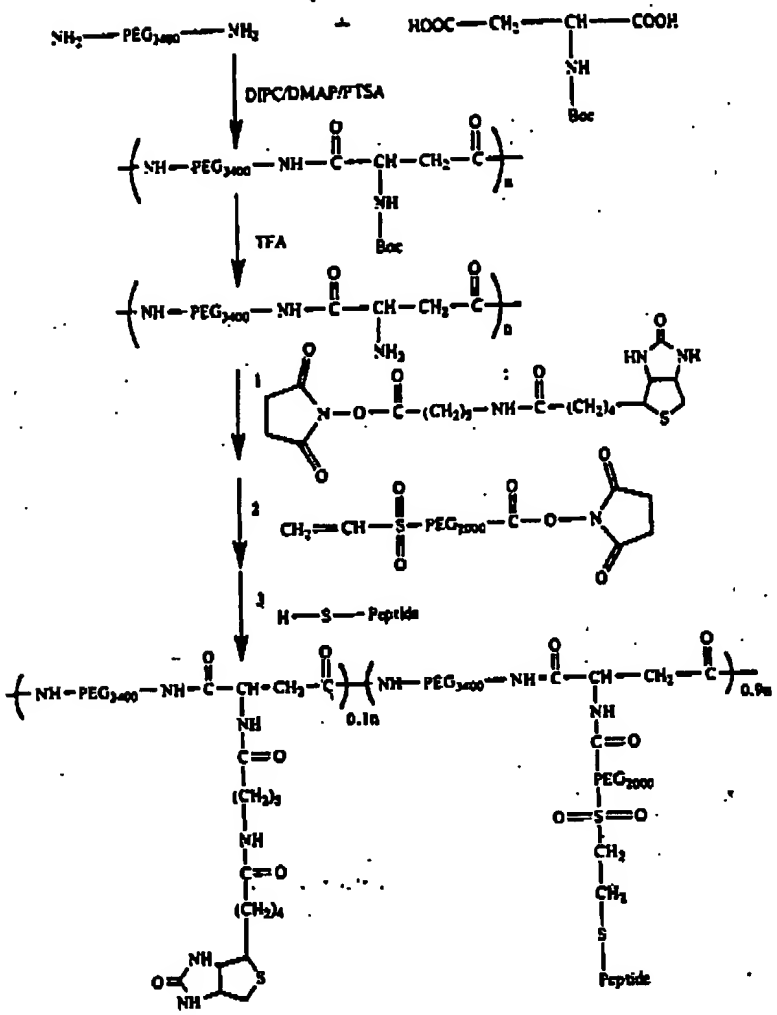


FIGURE 4 Synthetic scheme of biotin–PEG–epitope conjugates.

epitope peptides to the unaltered ELISA plates. The carriers are selected from proteins, such as bovine serum albumin (BSA), mouse serum albumin (MSA), rabbit serum albumin (RSA), keyhole limpet hemacyanin (KLH), and ovalbumin.[17] The conjugation of epitope peptides to these protein carriers is normally achieved through free amino groups present on the surface of the carrier proteins. For example, BSA has 59 lysine, and 30–35 are exposed to the protein surface and available for coupling. The advantages of using this kind of conjugate are as follows: (1) Not one single copy but multiple copies of epitope peptides can be immobilized to the plates through the carrier proteins. (2) Immobilized epitope peptides can assume favorable orientation and conformation for strong antibody binding. (3) Multiple copies of epitope peptides can considerably increase the avidity of epitope peptides to serum antibodies due to multivalent antigen–antibody binding. The protein carriers work well for most direct immunoassays and have been widely used to prepare various conjugates. However, there is a potential problem for them to be used in an indirect immunoassay, which is their high tendency of adhering directly to the plates. When using protein–peptide conjugates as the antigen source to detect antibodies in an indirect immunoassay, the carrier protein itself may adhere to the plates causing non-specific binding and elevated background. This problem is particularly significant in developing diagnostic assays for early stage LD, because the antibody level is relatively low and the signals are barely detectable at early stages. The elevated background could compromise the signals and possibly ruin the sensitivity and the specificity of the assays.

We designed a new PEG–peptide conjugate system with multiple copies of epitope peptides to achieve a

Table II Peptide Copy Numbers of Biotin–PEG–Peptide Conjugates by Amino Acid Analysis[a]

Biotin–PEG–Peptide Conjugate	β-Ala (pmol)	Asp from Polymer (pmol)	Peptide Copy Number
Biotin–PEG–FLA	388	282	6.0
Biotin–PEG–OspC-2	462	604.5	3.0
Biotin–PEG–OspC-3	141	192.5	3.0
Biotin–PEG–OspC-10	138.6	218	3.0
Biotin–PEG–P83-1	374	289	5.0
Biotin–PEG–P83-3	552.5	394.6	6.0
Biotin–PEG–P39	282	177	6.0

[a] Peptide Copy Number = (β-Ala/2)/(Asp from polymer/8).

strong and stable antigen–antibody binding for the selected epitope peptides. There are many advantages of using PEG–peptide conjugates as antigen source in an immunoassay, including what follows: (1) The nonimmunogenic PEG carrier significantly lowers assay background by avoiding any nonspecific binding to the surface of the plates, which is observed with other commonly used carrier proteins. (2) The multiple copies of epitope peptides in the conjugates favor multivalent interaction between the epitope peptides and two or more Fab fragments of the antibody for greater avidity. (3) The gentle reaction required for the conjugation would not affect the sensitive amino acids, such as Asp, Glu, and Lys, in the sequence of the epitope peptides. (4) The large molecular weight of PEG makes it very easy to separate the final conjugates from excess peptides and other small size impurities. (5) The inert PEG carrier does not aggregate, degrade or denature like other protein carriers do; thus the PEG–peptide conjugates are more stable and more convenient to be used in diagnostic assays. (6) The multiple attachments of reporter group to PEG carrier and the N-terminus of epitope peptides further amplify the assay signal.

In order to apply a highly sensitive biotin–avidin detection system in ELISA, biotin was first attached to a portion of the primary amines in the PEG copolymer pendant chains by reacting the copolymer with N-hydroxysuccinimide long-chain biotin (NHS–LC–biotin). The extended spacer arm reduces steric hindrance and ensures effective binding between the biotin molecules of the conjugates and the avidin–enzyme complex. Based on stoichiometric calculation, about 10% of amino groups were designed to attach biotin molecules. The remaining 90% of amino groups were reserved for epitope peptide conjugation. It is important to control the extent of above reactions to obtain the desired PEG–peptide conjugate, which needs to contain multiple copies of epitope peptides for stronger antibody–antigen interaction and sufficient biotin molecules for biotin–avidin detection. To achieve this goal, a fluorometric assay using fluorescamine was employed to monitor the reaction process so that the extent of biotinylation could be controlled precisely.[21] Since the fluorescence is proportional to the primary amine concentration, the reaction percentage of the amino groups in the PEG copolymer can be calculated based on fluorescence readings.

To conjugate epitope peptides to the polymer backbone, a two-step approach was used. A heterobifunctional cross-linker, NHS–PEG–VS was first reacted with the reserved amino groups in the reporter-labeled polymer carrier through the NHS groups. After removing excess cross-linker, cysteine-containing epitope peptides can then react readily with vinylsulfone groups (VS) to complete the conjugation. The final PEG–peptide conjugates containing multiple copies of epitope peptides and several copies of reporter molecules are now ready for immunoassays (Figure 4). The peptide copy numbers of the conjugates were determined by amino acid analysis (Table II). The calculation was based on the β-alanine in epitope peptides and aspartic acid in PEG–aspartic acid copolymer, assuming an average of 8 residues of Asp in the copolymer backbone. The formula for calculation of the peptide copy number is

$$\text{Peptide Copy Number} = (\beta\text{-Ala}/2)/(\text{Asp in polymer}/8)$$

ELISA Format Design

ELISA is a simple but very sensitive immunoassay involving the following basic steps: An antigen is bound to a solid phase material, usually a 96-well plastic plate, and the solution containing the antibody to be detected (usually serum) is added to the well having the immobilized antigens. After unrelated, unbound antibody is washed away, a second antibody, which is an anti-immunoglobulin antibody linked with an enzyme, is added to the wells. Then the substrate for the enzyme is added to the above reaction mixture and the amount of enzymatically altered substrate is measured. The enzyme and substrate are chosen so that enzymatic modification of the substrate produces a change in color of the substrate solution. The amount of changed substrate (which is quantitated spectrophotometrically) is proportional to the

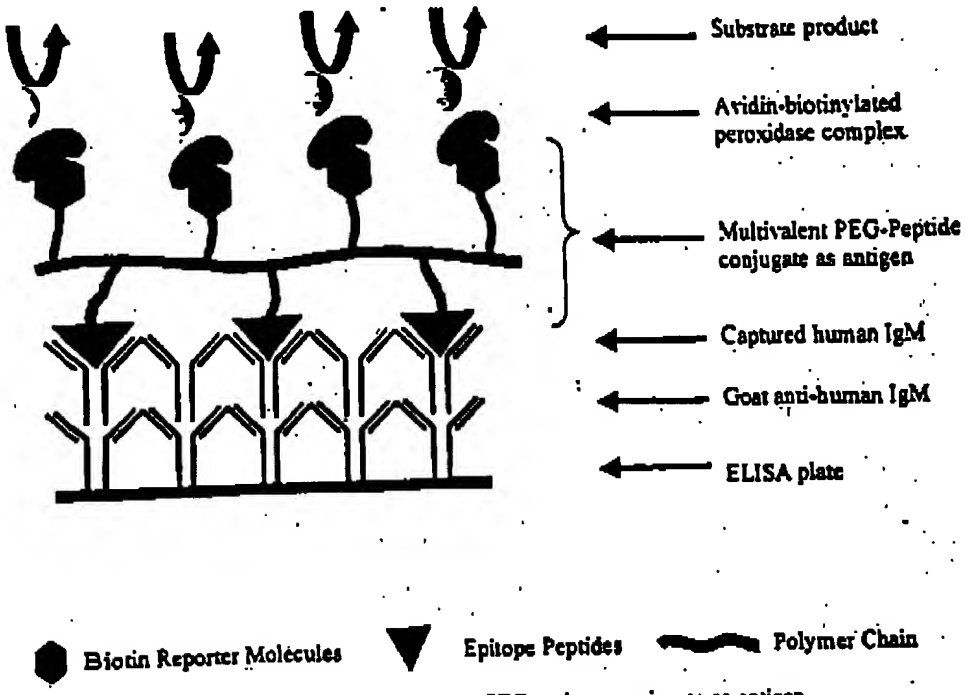


FIGURE 5 IgM-capture ELISA using biotin–PEG–epitope conjugate as antigen.

amount of antibody bound to the immobilized antigen.

Our goal is to develop an immunoassay for the diagnosis of early stage LD. Typically, the Lyme-specific immunoglobulin M (IgM) antibody first develops within 2–4 weeks after the infection, peaks after 6–8 weeks, and then declines after 4–6 months of illness in most patients. Elevated IgM antibody persists in LD patients and does not come down to normal range.[22] The Lyme-specific immunoglobulin G (IgG) antibody usually occurs within 6–8 weeks after onset of infection, peaks after 4–6 months, and may remain elevated indefinitely. Since the IgM antibody is the initial immune response, it makes sense to test this antibody specifically for the earliest diagnosis. Because there are many times more IgG antibodies (not necessarily Lyme specific) than IgM present in serum, they may interfere with the detection of IgM antibodies. Furthermore, the problem is compounded by the fact that the IgG antibodies are generally of much higher affinity and so may disproportionately inhibit by competition of the binding of IgM antibodies to the antigen.

In our assay development, we decided to use an indirect, IgM-capture ELISA format, in which only IgM antibodies are captured in the anti-IgM coated well and all other classes of antibodies are removed. Unlike a direct ELISA in which the antigens are immobilized on the surface of the well, an indirect class-capture ELISA refers to an assay format in which the antigens are present in the test solution to interact with the captured antibodies. It should be noted that a representative portion of all IgM antibodies, including LD-specific and unrelated IgM antibodies, are captured in the antibody capture step. When the captured IgM antibodies are exposed to the prepared PEG–peptide conjugates, these LD-specific epitope conjugates will only bind to LD specific IgM antibodies. If no LD-specific IgM antibodies are present, all conjugates will be washed away and no signal can be detected. As a result, a negative result is obtained. Clearly, this indirect IgM capture ELISA format, combined with using the LD-specific conjugates as antigens, will largely increase the sensitivity and the specificity of detecting LD-specific IgM antibodies, on which a highly sensitive and specific immunoassay can be developed.

Figure 5 demonstrates the indirect IgM-capture ELISA format for biotin–PEG–peptide conjugates. The plate was first coated with goat antihuman IgM antibody that can capture a representative portion of all IgM antibodies presented in test sera, including both Bb-specific and non-Bb-related antibodies. Next, the biotin–PEG–peptide conjugates were added to each well to interact with the captured IgM antibodies. Only Bb-specific IgM antibodies can react with the conjugates through multiple copies of epitope peptides in the conjugates. When tested with control

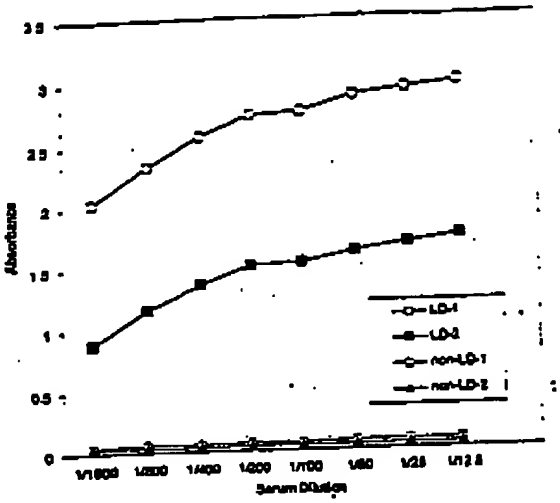


FIGURE 6 Serum dilution curve for biotin-PEG-peptide conjugates

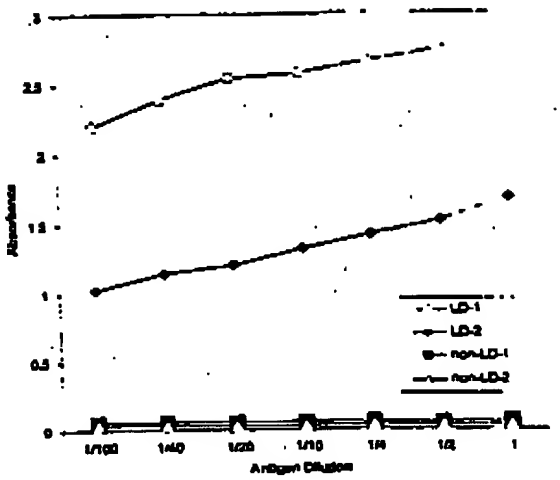


FIGURE 7 Antigen dilution curve for biotin–PEG–peptide conjugates.

sera, no Bb-specific IgM antibodies were present: the captured non-Bb-related IgM antibodies will not interact with epitope peptides. Therefore the conjugate antigens were simply washed away and no signal could be detected. On the other hand, when tested with LD-positive sera, Bb-specific IgM antibodies were present and captured from the test sera. They interacted with epitope peptides and retained the conjugates for continuing signal detection. The labeling biotin molecules were now available for interaction with avidin–biotinylated peroxidase and to yield a positive signal when treated with the substrate solution.

To optimize ELISA conditions for clinical studies, serum dilution and antigen dilution curves were determined by testing two Lyme-positive and two Lyme-negative sera. For serum dilution curve determination, a series of 2-fold diluted serum samples were added to wells of the ELISA plates. As shown in Figure 6, the absorbance for Lyme-positive serum samples increased as the final serum concentration increased from 1:1600 to 1:10 dilution, while the absorbance for the negative samples did not change very much. A serum dilution of 1:10 was selected to achieve the largest difference between the positives and the negatives. For antigen dilution curve determination, a series of diluted antigen samples were added to wells of the ELISA plates. The absorbance for Lyme-positive serum samples increased as the final antigen concentration increased from 0.01 to 1 μg/well, while the absorbance for the negative samples stayed low (Figure 7). PEG–peptide conjugates were applied in ELISA as antigens with a dilution of 0.1 μg/well for each conjugate to obtain sufficiently high absorbance for positives but maintain relatively low absorbance for negatives. In addition, the time course of color development was also determined as shown in Figure 8. The color intensity increased quickly in the first 10 min, but the curve flattened after that time to reach the maximum at 60 min. The color development time of 10 min was chosen in our clinical studies to yield accurately detectable signals within a reasonably short time period.

A panel of serum samples consisting of sera from subjects with and without LD was tested in the IgM-capture ELISA using biotin–PEG–peptide conjugates as the antigens. Sera with the prefix MC were ob-

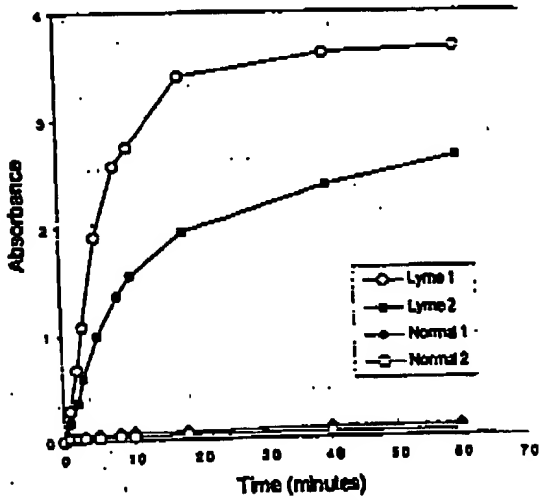


FIGURE 8 Time course of color development for biotin–PEG–peptide conjugates.

tained from patients with EM who have been shown to be positive by culture of the *Bb* spirochete. Sera with the prefix NC were obtained from individuals without a history of current or past infection with LD and were used as negative samples in this study. Syphilis serum samples were collected from patients with well-diagnosed infection by the syphilis spirochete. The general procedure for IgM-capture ELISA was similar as described above, but the assay conditions were optimized in terms of serum dilution, antigen dilution, and color development time. A group of 12 negative control sera were tested under the same assay conditions and the average absorbance plus three standard deviations of these control serum samples was used as the cut-off. The index number of each serum sample was calculated as

$$\text{Index number} = \text{Absorbance of individual serum}/\text{Cut-off}$$

An index number of 1.0 or above is taken as a positive and an index number of 0.8 or below is taken as a negative. Any index number between 0.8 and 1.0 is taken as equivocal.

The ELISAs of each individual PEG–peptide conjugate and the combination of all seven conjugates are conducted. Out of a total of 33 culture-proven positive serum samples, OspC-3, OspC-10, and P83-1 each detected 25 (25/33 or 76%), OspC-2 and P83-3 each detected 26 (26/33 or 78%), P39 detected 27 (27/33 or 82%), Fla detected 28 (28/33 or 85%), and the combination of all seven conjugates detected 31 (31/33 or 94%). It is clear that antibody responses to seven PEG–peptide conjugates are different and the combination of all seven conjugates detected more positives than any individual conjugate, indicating that the combination test format may have higher potential to be developed into an excellent diagnostic assay. For many sera, the index numbers varied with each individual PEG–peptide conjugate, and higher index numbers were observed for the combination of all seven conjugates. Clinically, this is very important because many serum samples would have been negative or equivocal when tested only with individual conjugates, as with sample MC-8, MC-33, and MC-71, but they would be correctly identified as true positives if the combination formats were used. Two samples, MC-9 and MC-68, were supposed to be Lyme positive according to culture results, but failed to be picked up even by the combination of all seven PEG–peptide conjugates. These samples were also tested negative in other LD diagnostic assays done previously, possibly because they were taken in a very early stage of the infection even before the antibodies developed to a detectable level in the patients. It has been found that it generally takes 2–4 weeks for IgM antibodies to reach a detectable level after initial infection of LD.[23,24] In fact, this phenomenon was observed in our study by testing two serum samples taken from the same patient at two different time points. The MC-3 (6/18) sample was tested negative while the MC-3 (6/26) sample taken one week later was tested strongly positive, suggesting that a two-test strategy might be able to achieve higher assay sensitivity in LD diagnosis. When a patient is tested negative in the first test, it is highly recommended to have a second test one or two weeks later before making a negative diagnosis. However, we have not done a study with sera taken at different time points after the tick bite to see how early our test can detect LD-specific IgM.

For comparison, the results from a protein-based (*Bb* sonicate) ELISA and our peptide-based ELISA, as well as the clinical diagnosis of a panel of sera, are listed in Table III. The peptide-based ELISA using the combination of seven conjugates identified 31 positive samples from 33 culture-proven positive samples, resulting in a diagnostic sensitivity of 94% (percentage of disease samples correctly diagnosed). The protein-based ELISA using sonicated *Bb* spirochete picked up 23 samples out of 31 tested positive sera, yielding a diagnostic sensitivity of 74%. Furthermore, the peptide-based ELISA did not yield any false positive results with the non-LD samples giving an essentially 100% of diagnostic specificity, whereas the protein-based ELISA gave 6 false positives out of 23 negative samples, or a diagnostic specificity of 74% (percentage of nondisease samples correctly diagnosed). Thus, the peptide-based ELISA achieved higher sensitivity and specificity than the protein-based ELISA.

As our design rationale predicted, the defined epitope peptides should have less chance compared with whole proteins to cross-react with sera from patients with other diseases. In order to further examine this hypothesis, a panel of serum samples from patients with syphilis infection was tested using the combination of PEG–peptide conjugates (Table IV). Indeed, while 13 out of 25 syphilis samples gave cross-reactive results in the protein-ELISA, none of these tested syphilis samples showed cross-reactivity in our peptide-ELISA when corrected by subtracting serum background (no antigen used in ELISA), indicating that all seven epitope peptides defined in this study are LD specific and do not cross-react with antibodies against the syphilis spirochete.

Table III Comparison of Lyme Disease Diagnosis for a Panel of Serum Samples[a]

No.	Protein ELISA	Peptide ELISA	Clinical Diagnosis	No.	Protein ELISA	Peptide ELISA	Clinical Diagnosis
MC-2	P	P	P	NC-1	P	N	N
MC-3 (6/18)	N	P	P	NC-2	N	N	N
MC-3 (6/26)	P	P	P	NC-3	N	N	N
MC-4	P	P	P	NC-4	N	N	N
MC-7	P	P	P	NC-5	N	N	N
MC-8	N	P	P	NC-8	N	N	N
MC-9	P	E	P	NC-9	N	N	N
MC-10	P	P	P	NC-10	N	N	N
MC-14	P	P	P	NC-11	N	N	N
MC-17	P	P	P	NC-14	N	N	N
MC-23	P	P	P	NC-15	N	N	N
MC-33	P	P	P	NC-16	N	N	N
MC-41	P	P	P	NC-A	N	N	N
MC-59	P	P	P	NC-B	N	N	N
MC-62	P	P	P	NC-C	P	N	N
MC-68	N	E	P	NC-D	N	N	N
MC-70	P	P	P	NC-E	P	N	N
MC-71	E	P	P	NC-F	N	N	N
MC-72	P	P	P	NC-G	N	N	N
MC-73	P	P	P	NC-H	N	N	N
MC-74	N	P	P	NC-LT	P	N	N
MC-91	N	P	P	NC-LN	P	N	N
MC-92	P	P	P	NC-LP	P	N	N
MC-93	N	P	P	MC-SC	P	P	P
MC-100	N	P	P	MC-EL	P	P	P
MC-101	P	P	P	MC-AN	P	P	P
MC-JS	P	P	P	MC-MT	ND	P	P
MC-GR	P	P	P	MC-HA	ND	P	P

[a] P, positive; N, negative; E, equivocal; ND, not determined.

EXPERIMENTAL

Epitope Selection by SPOTs Method

All concurrent peptide sequences were generated using computer software provided by the manufacturer with the SPOTs kit. By providing a protein sequence, desired length of peptides and offset of amino acids for each peptide, the program can edit peptide sequences to be assembled on SPOTs membrane and provide the amino acid addition schedule for each synthesis cycle. To start the peptide synthesis on the membrane, pre-weighed Fmoc-amino acid active esters were dissolved in dimethylformamide (DMF) and pipetted to appropriate spots on the membrane based on the generated synthesis schedule. Double coupling was done for each cycle to ensure the completion of the reaction. All the Fmoc-amino acid active esters, except arginine, are relatively stable and can be dissolved in DMF for use of several cycles in the same working day, as long as they are stored at −20°C between each addition. Due to its intrinsic instability, the Fmoc-arginine active ester must be dissolved just before use and a fresh aliquot must be used for each coupling cycle. The initial color of all spots on the membrane was blue, which is produced by bromophenol blue in the presence of the free amino groups on the deprotected amino acids. As coupling proceeds with the addition of Fmoc-amino acid active esters, the spots change to different colors for different amino acids. For example, asparagine and threonine change to green, serine changes to yellow. The color change can be regarded as a sign that the coupling is taking place. The membrane was washed with 3 × 20 mL of DMF for 2 min each time to remove excess active esters. Then acetic anhydride was added to acetylate any uncoupled amino groups to ensure no formation of deletion sequences. Since all free amino groups are capped by acetylation, the remaining blue color disappeared. The membrane was washed 3 × 20 mL DMF, and then 20 mL of 20% piperidine in DMF was added to remove Fmoc protecting groups. After washing the membrane with 5 × 20 mL DMF, 200 μL of 1% bromophenol blue solution was added to 20 mL DMF, and this solution was added on the membrane. Due to piperidine removal of the Fmoc groups, the spots turned blue, leaving the surrounding membrane white and

Table IV Comparison of ELISA Results for Serum Samples from Patients with Syphilis[a]

No.	Protein ELISA	Peptide ELISA	Clinical Diagnosis
S-1	CR	NCR	Syphilis
S-2	CR	NCR	Syphilis
S-3	CR	NCR	Syphilis
S-4	CR	NCR	Syphilis
S-5	CR	NCR	Syphilis
S-6	CR	NCR	Syphilis
S-7	CR	NCR	Syphilis
S-8	NCR	NCR	Syphilis
S-9	CR	NCR	Syphilis
S-10	NCR	NCR	Syphilis
S-11	NCR	NCR	Syphilis
S-12	NCR	NCR	Syphilis
S-13	CR	NCR	Syphilis
S-14	NCR	NCR	Syphilis
S-15	NCR	NCR	Syphilis
S-16	NCR	NCR	Syphilis
S-17	NCR	NCR	Syphilis
S-18	NCR	NCR	Syphilis
S-19	CR	NCR	Syphilis
S-20	CR	NCR	Syphilis
S-21	CR	NCR	Syphilis
S-22	CR	NCR	Syphilis
S-23	NCR	NCR	Syphilis
S-24	NCR	NCR	Syphilis
S-25	NCR	NCR	Syphilis

[a] CR, cross-reactive; NCR, noncross-reactive.

the solution yellow. The membrane was washed with 3 × 20 mL of methanol. After air drying on a sheet of folded filter paper, the membrane is ready for the next coupling cycle. This procedure was repeated for all but the final coupling cycle of the synthesis. For the final cycle, piperidine treatment was carried out right after the double coupling of active esters and DMF washing. Bromophenol blue solution was then added to obtain blue color for all spots, and finally the peptides on each spot were capped by acetylation. After synthesis and acetylation, the protecting groups present on the side chains of the amino acids must be removed. For side chain deprotection, 5 mL of dichloromethane (DCM) was mixed with 5 mL TFA. The mixed solution was added immediately onto the air-dried membrane and the cleavage reaction was allowed to proceed for 1 hour. The membrane was then washed with 3 × 20 mL DCM, 3 × 20 mL DMF, and 3 × 20 mL methanol. The membrane was air-dried and stored in a sealed plastic bag in the freezer (−20°C) until required for SPOTs analysis.

The SPOTs membrane was first blocked with 20 mL of TBS-blocking buffer overnight at room temperature. The membrane was washed with 20 mL TBS containing 0.05% Tween-20 (T-TBS). The serum sample (LD or control) was diluted in 20 mL TBS-blocking buffer to 1:100. This diluted test antibody solution was added to the membrane and rocked for 3–4 h at room temperature. The membrane was washed with 3 × 20 mL T-TBS for 10 min each wash. Then 100 μL of β-galactosidase conjugated antihuman (G + M + A) secondary antibody was diluted with 20 mL of TBS-blocking buffer. This was added to the membrane and rocked for 2 h at room temperature. During this time, the signal development solution was prepared as follows: The amount of 4.9 mg 5-bromo-4-chloro-3-indoyl-D-galactopyranoside (BCIG) was dissolved in 100 μL DMF and 100 mg potassium ferricyanide in 1 mL MilliQ water. The BCIG solution and 100 μL of potassium ferricyanide solution were added to 10 mL of phosphate buffered saline (PBS) containing 10 μL of 1*M* magnesium chloride solution. After the incubation of the secondary antibody solution, the membrane was washed with 2 × 20 mL T-TBS followed by 2 × 20 mL PBS; then the prepared signal development solution was added to the membrane and rocked at room temperature until blue spots appear. The color was allowed to develop for 40–50 min until a point at which there is a clear distinction between positive and negative spots. The signal development solution was poured off and the membrane washed with 2 × 20 mL PBS. The stained membrane was photographed to provide a permanent record.

The SPOTs membrane must be regenerated after analysis of each serum sample to remove bound proteins before storage or reprobing. To regenerate the membrane, it was washed with 5 × 20 mL MilliQ water and then 3 × 20 mL DMF followed by another 2 × 20 mL MilliQ water. Then 20 mL of regeneration buffer A (485.0 g urea, 10.0 g SDS, and 1 mL 2-mercaptoethanol in 1 L of MilliQ water) was added and the membrane was incubated for 10 min at room temperature. The process was repeated twice. Then 20 mL of regeneration buffer B (mix 400 mL of MilliQ water and 500 mL ethanol; add 100 mL of acetic acid to above solution) was added and the membrane was incubated for 10 min at room temperature. The process was repeated twice. Finally, the membrane was washed with 2 × 20 mL methanol and air-dried. The membrane was stored in a sealed plastic bag in the freezer (−20°C) until the next analysis.

Synthesis, Purification, and Characterization of Epitope Peptides

All seven epitope peptides were synthesized by the solid phase technique using Fmoc chemistry. PAL solid support was used in the synthesis to obtain C-terminal amide upon cleavage for all epitope peptides. DMF (3 mL) was added to swell the resin for 20 min. After Fmoc deprotection with 20% piperidine in DMF for 2 × 20 min, the resin was rinsed with 3 mL of DMF three times, 3 mL of methanol three times, then dried in air. The coupling was achieved by adding 3-fold molar excess of each amino acid, mixed with equimolar amounts of benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and HOBt in 3 mL of DMF containing 1% (v/v) diisopropylethylamine (DIEA). Coupling proceeded at room temperature for 4 h. After coupling, the resin was washed with DMF

and methanol and air-dried. A sample of the resin was tested with Kaiser ninhydrin reagent (1:1:1 v/v/v 0.2 m*M* KCN in pyridine, 4 mg/mL of phenol, and 5% ninhydrin in butanol) at 110°C for 3 min.[26] If the resin showed blue color, double coupling would be conducted for another 4 h to drive the reaction to completion. The resin was capped using 4 mL of DMF, 400 μL of acetic anhydride, and 80 μL of triethylamine for 4 h to eliminate any unreacted amino groups. The coupling procedure was repeated until the desired peptide sequence was obtained. When the assembly of the peptide sequence was complete, the N-terminus of the peptide was acetylated with 2% of acetic anhydride in 1% of DIEA/DMF (v/v). All peptides were cleaved from the resin with TFA/thioanisole/ethanedithiol (EDT)/anisole (90/5/3/2%, v/v) at 1 mL/100 mg resin for 2 h at room temperature. The cleavage mixture was filtered through glass wool, which was then rinsed with TFA twice. The filtrates were combined and evaporated under an Argon stream to reduce the volume to about 1–2 mL, then precipitated by adding dropwise into 10 times volume of ice-cooled diethyl ether. The white precipitate was washed with cold diethyl ether five times to remove scavengers. Crude peptide was then dried under vacuum overnight and stored under Argon in the freezer (−20 °C).

Synthesis and Purification of PEG–Aspartic Acid Copolymers

Amino group protected L-aspartic acid (Boc-Asp-OH) and α,ω-diamino-PEG (NH_2-PEG-NH_2, MW 3,400 Da) were copolymerized based on carbodiimide reaction in the presence of DMAP and PTSA as catalysts. In a typical preparation, NH_2-PEG-NH_2 (680 mg, 2×10^{-4} mol) and Boc-Asp-OH (46.6 mg, 2×10^{-4} mol) were dissolved in 20-mL methylene chloride with stirring. DMAP (12.2 mg, 1×10^{-4} mol) and PTSA (19.0 mg, 1×10^{-4} mol) were added. To this solution 1,3-diisopropylcarbodiimide (DIPC) (15.6 mL, 1×10^{-3} mol) was added at 0°C under stirring. The reaction flask was sealed with a rubber stopper assembled with an argon balloon. The reaction was allowed to continue at room temperature with stirring until the reaction mixture became viscous. The reaction mixture was precipitated in 10 volumes of ice-cooled ethyl ether to obtain the white polymer product. The polymer was washed three times with ice-cooled ethyl ether and the polymer product was collected by filtration or centrifugation. The polymer was dried under an argon flow, redissolved in MilliQ water and purified by dialysis using Spectra/Por, spectrum cellulose ester membrane (MWCO 12–14,000 Da) for 24 h. After lyophilization, the polymer was treated with TFA for 3 h to remove all the Boc protecting groups. The deprotected polymer solution was then precipitated in 10 volumes of ice-cooled ethyl ether, washed three times with ice-cooled ethyl ether and dried under vacuum. The molecular weight of the resulting PEG copolymer was measured by SEC.

Preparation of PEG–Peptide Conjugates

To a solution of PEG copolymer in 50 m*M* carbonate–bicarbonate buffer (pH 8.5) was added 0.5 equivalent (relative to the amino groups in the polymer) of NHS-LC-biotin in DMSO. The mixture was stirred at room temperature under argon overnight. After about 10 h of reaction, approximately 30% of the amino groups in the PEG copolymer were reacted and linked to biotin molecules. A fluorometric assay, using a fluorogenic reagent, Fluram, was employed to check the extent of the biotinylation reaction. To describe briefly: 100 μL of PEG copolymer solution was saved before adding the biotinylation reagent and diluted 10× in 0.2*M* borate buffer (pH 8.5) as reference. When reaction was complete, 100 μL of reaction mixture was taken and diluted 10× in 0.2*M* borate buffer (pH 8.5) as sample. For fluorometric assay, 50 μL of Fluram solution (15 mg Fluram dissolved in 25 mL acetonitrile) was added to 150 μL of diluted reference, 150 μL of diluted sample and 150 μL of blank 0.2*M* borate buffer, pH 8.5), respectively, in separate wells of a microtiter plate. After mixing immediately by pipetting up and down several times, fluorescence was read on a Fluorescence Multi-Well Plate Reader (CytoFluor II, PerSeptive Biosystems) with the excitation wavelength set at 400 nm and the emission wavelength set at 460 nm. The biotin labeled PEG copolymer was purified by a Pharmacia Superdex-75 column and then reacted with 3 molar equivalents of heterobifunctional NHS-PEG-VS (MW 2000 Da), relative to free amino groups remaining in biotin-labeled PEG copolymer. The latter reaction, which was also monitored by the fluorometric assay, was complete after 4 h at room temperature (25 °C). The fluorometric assay procedure was similar to that described above. The final fluorescence reading was equal or close to the blank reading, suggesting that all amino groups in the PEG copolymer had been successfully derivatized. The reaction product was purified through a Pharmacia Superdex-75 column or by membrane dialysis. For peptide conjugation, 5 molar equivalents of peptide relative to the available VS groups in the PEG copolymer were added to the activated polymer solution, and these were allowed to react at 4°C overnight. The final biotin-PEG-peptide conjugate was purified by the Pharmacia Superdex-75 column or by membrane dialysis, and concentrated to about 1 mg/mL using Centricon ultrafilter (MWCO 10,000 Da). Aliquots were stored as the stock antigen solution in the freezer (−20°C) until needed.

IgM Capture ELISA

ELISA plates were coated with 100 μL/well of affinity-purified goat antihuman IgM antibody (10 μg/mL) in 0.04*M* carbonate–bicarbonate buffer, pH 9.6. Plates were slowly rotated on a Titer Plate Shaker (Lab-Line, Melrose Park, IL) for 2 h at room temperature, and kept at 4°C overnight. The plates were washed three times in a plate washer (ELP35, Biotek, Winooski, VT) with PBS-B [10 m*M* phosphate buffered saline, 0.15*M*+ sodium chloride, containing 0.1% bovine serum albumin (BSA)], blocked with 300 μL/well of PBS-B milk (PBS-B containing 5% nonfat dry milk) for 2 h at 37°C. Serum samples were diluted 1:100 in PBS-B milk, added at 100 μL/well and rotated at 300 rpm for 1 h. The

plates were washed four times with PBS-B and incubated for 1 h with 100 μL/well of biotin-PEG-peptide conjugates (diluted to various concentrations in PBS-B milk). During this time, the avidin-biotinylated peroxidase complex (ABC) was formed by adding one drop (50 μL) of reagent A (avidin DH) and one drop (50 μL) of reagent B (biotinylated peroxidase) to 5 mL of PBS-BT (PBS-B containing 0.5*M* sodium chloride and 0.1% Tween 20). The ABC reagent was vortexed and kept at room temperature for at least 30 min before use. After washing the plates four times with PBS-B, 7 mL of PBS-BT was added to the ABC reagent and 100 μL of the diluted ABC reagent was added to each well. The plate was rotated at 300 rpm for 30 min and washed four times with PBS-B on the Biotek plate washer followed by two more manual washes with plain PBS. During the last wash, the two component 3,3',5,5'-tetramethylbenzidine substrate solution (TMB) was prepared at room temperature. Substrate was added at 100 μL/well with a repeater pipette (Eppendorf Plus/8), the plate was rotated for 10 min to develop the color, and the reaction was stopped by adding 100 μL/well of 1*M* phosphoric acid. The plate was then rotated for 2 more min to homogenize the color and then read on an ELISA plate reader (Biotek) set for dual wavelengths (450 and 630 nm).

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Exhibit B

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Introduction to Alginate

Structure of Alginate

Alginate was originally thought to consist of a uniform polymer of mannuronic acid as shown in figure 1. However later studies showed the presence of guluronic acid residues and it is now understood that alginate is a linear co-polymer of β-D-Mannuronic acid and α-L-Guluronic acid.

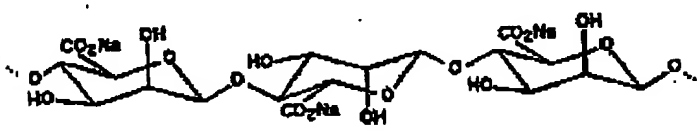

Figure 1. Sodium polymannuronic acid

Depending on the weed source and growing conditions the ratio of mannuronic and guluronic acid can vary. It is also known that the block structure within the alginate can vary significantly. The poly guluronic acid blocks bind significantly more effectively with calcium ions than the poly mannuronic acid blocks.

The weed types with the higher guluronic acid levels are normally the ones that show the strongest interaction with calcium and hence the strongest gel strength. However it is not quite that simple and only guluronic acid blocks over a certain size can be involved in calcium crosslinking and the larger the block the stronger the cross link. Hence to identify the alginate with the strongest calcium gel not only high guluronic acid levels are required but also significant block structures

Despite the stronger gel strength of the high guluronic acid containing weeds the major application for this product is in pet food. Most of the alginate sold into food and pharmaceutical applications today tends to be low in guluronic acid.

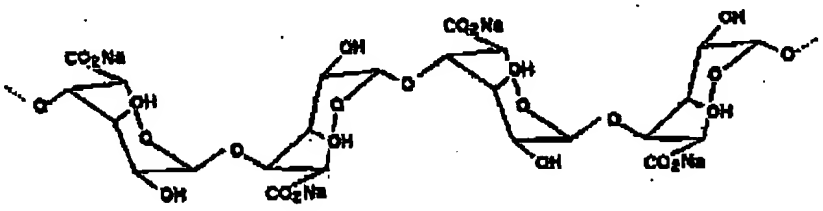

Figure 2. Sodium polyguluronic acid

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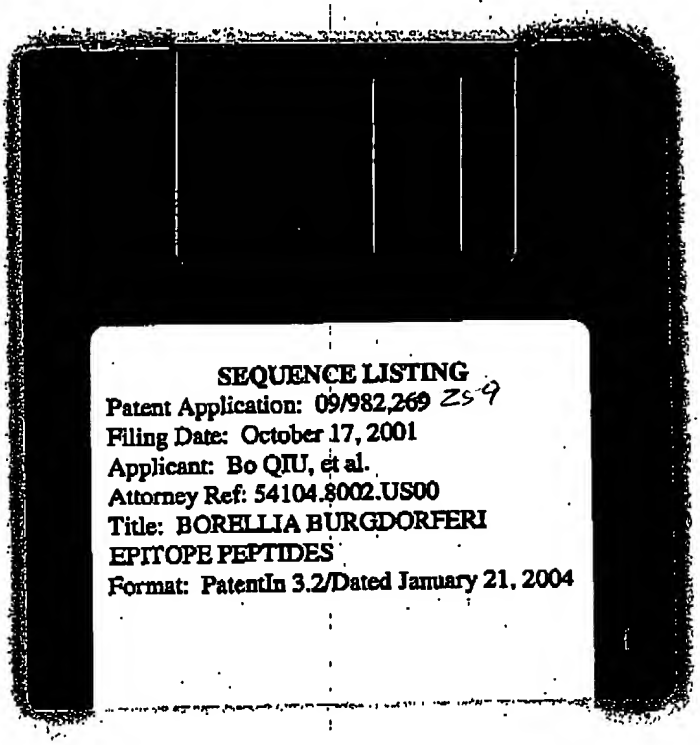
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Applicant: Bo QIU, et al.
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Title: BORELLIA BURGDORFERI
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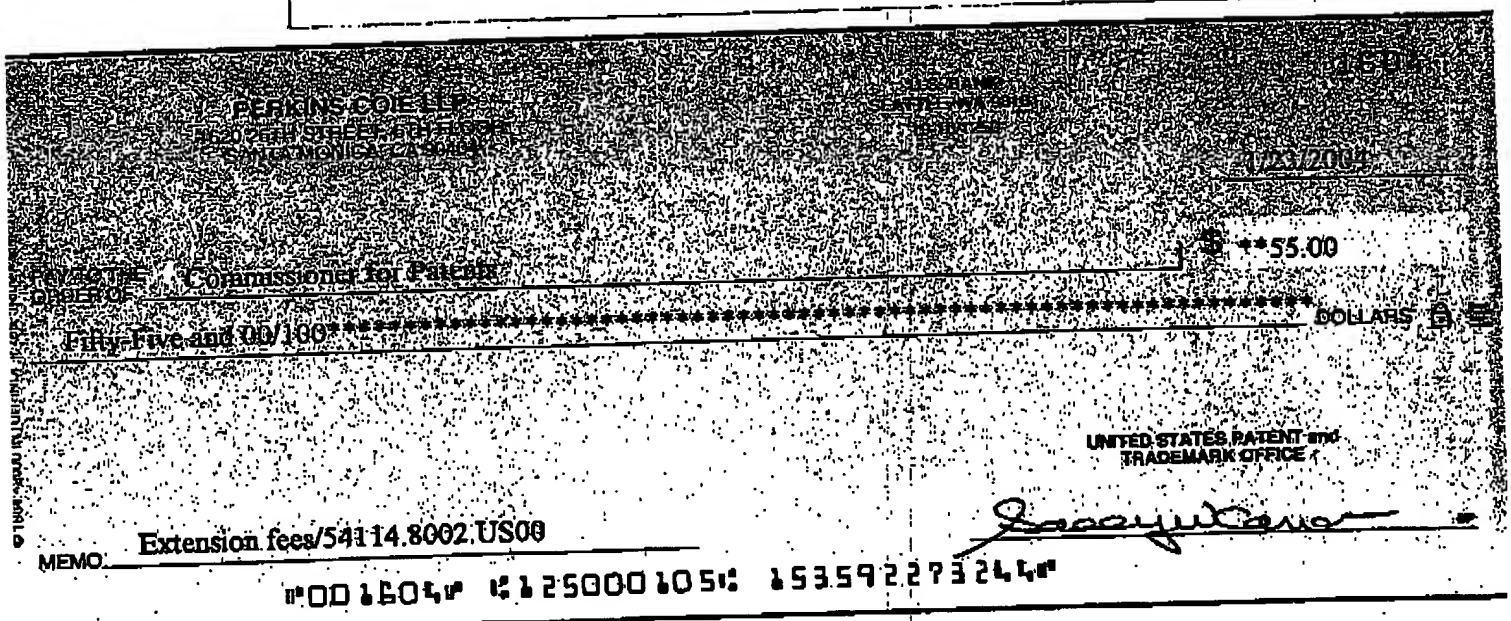

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